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Mini-Review

Metabolomic approaches for the characterization of fruits: a case study on avocado

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ABSTRACT

Metabolomics involves the study of the totality of metabolites present in the cell, tissue or whole organism in order to detect changes that occur in time and space. Various analytical tools, including gas and liquid chromatography, mass spectrometry, nuclear magnetic resonance spectroscopy and capillary zone electrophoresis, have been employed to characterize the metabolome of a sample, with each technique affording specific advantages and limitations. This review describes a range of metabolic studies that have been performed on fruit tissues in general, with emphasis on avocado in particular. Although avocado is of commercial importance throughout the world, it is a very disperse species and its chemical composition has been poorly studied, especially since nearly all investigations have focused on just one of its landraces. Metabolic profiling of avocado has aimed mainly at identifying biomarkers related to fruit growth, ripening, classification and storage, or has sought to establish the genetic architecture underlying the accumulation of metabolites within the fruit. The current state of knowledge concerning these diverse aspects is described herein, and possible pointers to the future trend in metabolomic studies involving fruit tissues are outlined.

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KEYWORDS: *Persea americana* Mill., avocado, metabolomics, fruit ripening, biomarkers, antioxidant activity

INTRODUCTION

Avocado (*Persea americana* Mill.; Lauraceae) is an arboreal species that originated from Mesoamerica and has been cultivated in central Mexico and some parts of the Guatemalan highlands before the arrival of the Spanish settlers. Although the wood, bark, leaves and seeds of the species are employed in a diverse array of applications, the fruits are especially prized by virtue of their culinary, nutraceutical and organoleptic properties. The fleshy mesocarp of the fruit is characterized by a high lipid content that varies in the range 3 to 30% by weight and is particularly rich in oleic, palmitic, linoleic and palmitoleic acids, but with only trace amounts of stearic acid (Table 1). Enhanced dietary intake of these fatty acids has been related to a reduction in the risk of cardiovascular diseases, apparently mediated by the antioxidant activities of these components and their capacity to preserve the levels of high-density lipoproteins [1-3].

Avocado fruits also contain high levels of bioactive lipophilic compounds including, among others, tocopherols (vitamin E), carotenoids and sterols that have been shown to possess antioxidant and radical scavenging activities [4]. Moreover,

Table 1. Nutrient composition of avocado.

Nutrient	**Amount/100 g**
Water	73.23 g
Energy	160 kcal
Protein	2.00 g
Total lipid (fat)	14.66 g
Fatty acids (total saturated)	2.126 g
Fatty acids (total monounsaturated)	9.799 g
Fatty acids (total polyunsaturated)	1.816 g
Fatty acids (total trans)	0.000 g
Total carbohydrate	9.19 g
Total dietary fiber	6.7 g
Minerals	
Calcium	12 mg
Iron	0.55 mg
Magnesium	29 mg
Phosphorus	52 mg
Potassium	485 mg
Sodium	7 mg
Zinc	0.64 mg
Vitamins	
Vitamin C (total ascorbic acid)	10 mg
Thiamin	0.067 mg
Riboflavin	0.130 mg
Niacin	1.738 mg
Vitamin B_6	0.257 mg
Folate (DFE)	81 µg
Vitamin B_{12}	0.00 µg
Vitamin A (IU)	146 IU
Vitamin E (α-tocopherol)	2.07 mg
Vitamin D (D2 + D3)	0.00 µg
Vitamin D	0 IU
Vitamin K (phylloquinone)	21.0 mg

Source: Adapted from Terry, L. 2011.

the polar fraction of the fleshy mesocarp contains sugars, amino acids, phenolic acids, flavonoids and polylphenols that help to enhance the functional properties of the fruit.

The characteristics of avocado fruits are influenced by a diverse range of factors that include the cultivar, agronomic conditions, post-harvest manipulation and the stage of ripeness [5]. In the commercial setting, ripening of the fruit does not occur on the tree but takes place after harvesting, typically within a period of 5 to 7 days at 25 °C [6]. The fruits have a high metabolic rate and the ripening process is dependent on the level of endogenous ethylene, augmentation of which increases the respiration rate and induces many physiological and biochemical changes, including the biosynthesis and accumulation of pigments, lipids, vitamins and antioxidants. However, relatively few studies have focused on alterations in the profiles of individual components during the process of ripening of the avocado fruit.

Metabolomics is an analytical tool that can be employed to gather information concerning the chemical composition of a plant tissue and relate this with its biological activity. While numerous articles describe the health-promoting and therapeutic properties of fruits and vegetables [7], including the identity, abundance and variance of fruits, as well as the bioavailability and efficacy of individual bioactive components derived therefrom, reviews concerning the metabolomic analysis of commercially important fruits are scarce. The aim of the present work is, therefore, to review the general trends in the metabolomic analysis of fruit ripening and development with special emphasis on avocado as a case study.

Metabolomic analysis of fruit development and ripening

Numerous metabolomic studies of diverse plant tissues have been published since early 2000, but papers relating to fruit development are relatively rare (see Table 2) and have focused mainly on ripening processes in tomato and strawberry [8]. In this context, the article by Tohge and Fernie [9] provides an excellent state-of-the-art review of the metabolomic analysis of tomato (*Solanum lycopersicum*) fruit with particular emphasis on compounds related to nutritional quality and shelf life. Regarding strawberry (*Fragaria* x *ananassa*

Table 2. Summary of the outcomes of metabolomic studies performed on fruits.

Fruit	Analytical method	Data analysis	Outcome	Reference
Tomato	GC-MS, LC-MS	PCA/PLS-DA	Understanding metabolic shifts during fruit development	[9]
Strawberry	GC-MS, LC-MS	PCA	Variation of phenolic compounds	[10]
Berries	DIMS-LC-MS	PCA	Differences in polyphenol content	[11]
Apple	GC-MS, LC-MS	PCA	Alteration of metabolome by pre-storage irradiation	[12]
Melon	GC-MS	PCA	Determination of aroma compounds	[13]
Peach	GC-MS	PCA	Detection of key factors related to traits	[14]
Pear	GC-ESI-TOF-MS	PCA	Metabolic flux analysis of browning disorders	[15]
Watermelon	^{1}H-NMR	PCA	Rapid analysis in breeding programs	[16]
Grape/Wine	LC-MS	O2PLS	Biomarkers of berry development and withering	[17]
Kiwi	UV-MS	ANOVA	Phenotype differences in total antioxidant capacities	[18]
Citrus	GC-MS, LC-MS	PCA	Biomarkers for fruit intake	[19]

^{1}H-NMR: proton nuclear magnetic resonance, ANOVA: analysis of variance, DIMS: direct infusion mass spectrometry, ESI: electrospray ionization, GC: gas chromatography, LC: liquid chromatography, MS: mass spectrometry, PCA: principal component analysis, PLS-DA: partial least squares discriminant analysis, TOF: time of flight, UV: ultraviolet.

Duch.) fruit, Zhang *et al*. [10] employed non-targeted (gas chromatography-mass spectrometry; GC-MS) and targeted (high performance liquid chromatography; HPLC) metabolic profiling together with principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to explore the profiles of non-polar and polar metabolites in fruit samples at seven developmental stages. Correlation of metabolites and application of network analysis revealed a degree of interdependence of the various metabolic pathways and individual metabolites. The ellagitannin and proanthocyanidin components present in tannin-enriched extracts of strawberries, raspberries (*Rubus idaeus*) and cloudberries (*R. chamaemorus*) have been investigated using LC-MS, MS/MS and direct infusion-MS [11]. The method provides a rapid, automatable, high throughput and scalable screen by which to assess phytochemical diversity of berry polyphenols, and is well suited for screening large number of samples in breeding programs.

Metabolomic analysis has been employed in studies aimed at furthering our knowledge concerning the post-harvest physiology of apple (*Malus domestica*) fruit. For example, Rudell *et al*. [12] investigated changes induced in the metabolome of the peel of apples that had been subjected to ultraviolet-white light irradiation prior to cold storage for six months. The authors detected distinct temporal alterations in the profiles of more than 200 compounds related to pre-storage radiation treatment, included

among which were various flavonoid pigments derived from the phenylpropanoid pathway.

Melons (*Cucumis melo*) are prized for their sweet and refreshing flesh and the volatile compounds that contribute to their characteristic aroma. Allwood *et al.* [13] carried out a detailed investigation of these volatile components using thermal desorption-GC-MS together with PCA, a strategy that allowed the discrimination of the studied cultivars. The authors claimed that this approach offers great potential for the assessment of aroma and quality of the fruit.

Metabolic profiling and analysis of the key regulatory enzymes of the juicy mesocarp of peach (*Prunus persica*) during developmental stages and subsequent post-harvest ripening revealed clear metabolic shifts [14]. The early developmental stages were characterized by decreased protein content but with high levels of polyphenols and amino acids. Moreover, the data indicated the existence of singular metabolic programs in the development of peach that could enable the key factors related to agronomic traits in this rosaceous species to be identified.

Pears (*Pyrus communis* cv. Conference) stored under low oxygen or high carbon dioxide conditions may develop core breakdown, a condition that is characterized by softening of the fruit and browning of the tissue caused by oxidation of phenolic components. Pedreschi *et al*. [15] carried out metabolic profiling on sound and browning tissue using GC coupled with electrospray ionization-time of flight-mass spectrometry (ESI-TOF-MS) and showed that the disorder arose from an imbalance of oxidative and reductive processes at the cellular level. The chemical profile of the brown tissue was clearly different from that of the sound tissue and showed a reduced level of malic acid and increased amounts of trehalose, putrescine and gluconic, γ-aminobutyric (GABA) and fumaric acids. It was concluded that the metabolic activity of the Krebs cycle was reduced in the brown tissue and that the GABA shunt pathway was likely blocked. The authors also provided the first indication that GABA and gluconic acid could be metabolic markers for core breakdown.

Metabolic profiling, with proton nuclear magnetic resonance (^{1}H-NMR) as the main analytical tool, has been employed by Jayaprakasha and Patil [16] to identify and quantify amino acids and sugars in the fruits of watermelon (*Citrullus vulgaris*). The authors noted that the method showed good specificity, linearity, accuracy, precision, reproducibility and robustness, and could be used to identify and quantify multiple compounds simultaneously in large numbers of biological or breeding samples.

Integration of 'omic' (i.e. transcriptomic, proteomic and metabolomic) data obtained during the development and post-harvest drying of grapevine (*Vitis vinifera*) berries allowed Zamboni *et al*. [17] to identify transcripts and proteins that were modulated during drying along with secondary metabolites that accumulated at the same time. Knowledge of such stage-specific networks will contribute to our understanding of the key molecular processes that determine the characteristics of wine.

Commercially grown fruits from various species of the genus *Actinidia* (kiwi fruit) are rich in vitamins, flavonoids and minerals and are considered excellent sources of antioxidants. Du *et al*. [18] evaluated eight genotypes of kiwi for antioxidant potential using a range of different assays, and determined their polyphenol composition and vitamin C content. Although the focus of the study was not on the use of the metabolomic tool *per se*, the contribution is valuable because the authors were able to show that both total polyphenols and vitamin C are the major contributors to the antioxidant capacity of the kiwi fruits.

An interesting study on the application of MS-based metabolomics in the discovery of biomarkers for the dietary intake of *Citrus* fruit juices has been described by Pujos-Guillot *et al*. [19]. Their novel approach involved HPLC-ESI-TOF-MS profiling of the urinary metabolomes of volunteers who had consumed acute doses of orange or grapefruit juice or orange juice regularly for one month. In both groups, *Citrus* consumption was reflected in the appearance of distinct signals, and various biomarkers were identified along with the already known markers, namely proline betaine and flavanone glucuronides. The authors suggest that metabolomics might eventually bring to light nutritional biomarkers that could be assessed in clinical samples in order to evaluate the dietary exposure of an individual.

Metabolomic analysis of avocado fruit

Only a few papers describe the application of metabolomics in the study of parameters related to the quality and characteristics of commercial avocado fruit. In this article we review and comment on the metabolomic and physiological data presented in a selection of articles published since 2000 to the present time. This selection was based on the review of more than two hundred papers, and just considered those related with the topic of this review.

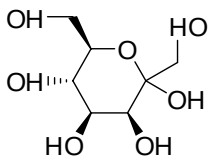
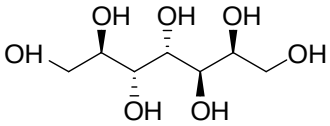
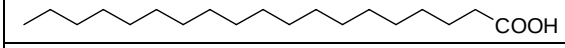
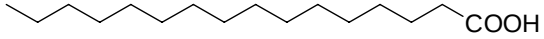
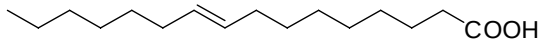
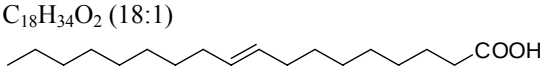
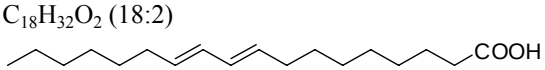
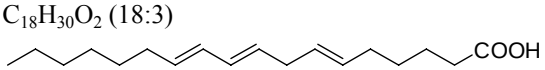
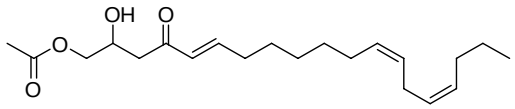
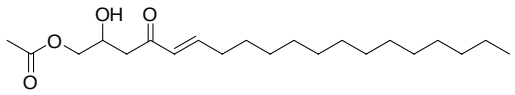
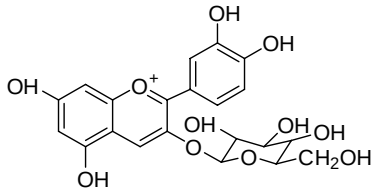
It is well understood that the perceived quality of avocado fruit depends on a combination of characteristics, attributes and properties, all of which have significance in product acceptability by the consumer. It is, therefore, important to determine the composition of the fruit in order to ensure high quality and to persuade the customer to purchase the product. Most avocado producers evaluate the oil content and/or dry matter of the fleshy pulp, comprising the mesocarp and the thin layer of endocarp, in order to establish harvestable maturity and acceptability of the fruit. A number of studies have focused on potential correlations between alterations in dry matter, oil content and fatty acid composition with stage of harvesting and the post-harvesting ripening period. For example, Ozdemir and Topuz [6] showed that changes in oil content and fatty acid composition differed depending on the variety of avocado, time of harvesting and the duration of post-harvest ripening. The authors considered that data related to the oil and fatty acid content of the fruit during maturation would be important in deciding the most suitable harvesting time.

Villa-Rodríguez *et al*. [20] evaluated the influence of the stage of maturation on the lipophilic and hydrophilic components in 'Hass' avocados and their relationship with the antioxidant properties of the fruit. The results showed that at the most advanced stage of ripeness (RS4), the oil content had increased up to 19.89% and the fatty acid content had attained 424.42 mg/100 g fresh weight. While total phenols tended to increase during ripening, with highest levels recorded in RS3 (31.88 mg gallic acid equivalents/100 g fresh weight), the total flavonoid content peaked at RS2 (26.36 quercetin equivalents/100 g fresh weight) and diminished at each ripening stage thereafter. Moreover, the antioxidant activity of the fruit increased with ripeness stage and correlated with the fatty acid content. These results confirmed the findings of an earlier study [5] by demonstrating that the stage of ripeness affected the physiological and physicochemical characteristics of the fruit as well as the content of bioactive compounds. Interestingly, some authors have found that later-harvested fruits tend to exhibit lower nutritional value and antioxidant activity after storage, and have suggested that early harvesting could afford economic advantages for the producer and greater health benefits for the consumer [21]. It has been noted that the pigments present in different parts of the fruit could also contribute to the antioxidant activity of avocado. In this context, it is reported [22] that the concentrations of carotenoids and chlorophyll in the skin and three sections of the flesh of 'Hass' avocados did not change significantly during ripening, while levels of anthocyanins in the skin increased when the fruit ripened and softened.

Ultra-HPLC coupled with ESI-TOF-MS has been employed to profile the metabolites in methanolic extracts of 13 varieties of avocado at two ripeness stages [23]. The sensitivity, precision and accuracy of the method were such that around 200 components in the extracts could be identified and quantified. The authors classified the data according to the different stages of ripeness, and employed a combination of non-supervised and supervised methods of multivariate analysis to delimit a set of compounds that were differentially regulated during the ripeness process. Table 3 shows the structures of some of the compounds detected in the fruit of avocado.

It is apparent from such studies that avocado fruits contain a complex matrix of components that can only be analyzed satisfactorily using robust methods. In this sense, Hurtado-Fernández *et al*. [24] have described the first application of a recently developed atmospheric pressure chemical ionization (APCI)-TOF-MS database to characterize the avocado fruit metabolome. The authors verified their results using the more traditional GC-EI-MS method and concluded that APCI represents a promising analytical tool considering the robustness and reliability of the technique and the wide range of polarities and sizes of analytes to which it may be applied.

Table 3. Structures of some compounds detected in avocado fruit.

Structure/formula	Name	Class	Reference
	Mannoheptulose	Carbohydrate	[34-36]
	Perseitol	Carbohydrate	[34-36]
$C_{18}H_{36}O_2$ (18:0) 	Stearic acid	Fatty acid	[3]
$C_{16}H_{32}O_2$ (16:0) 	Palmitic acid	Fatty acid	[3]
$C_{16}H_{30}O_2$ (16:1) 	Palmitoleic acid	Fatty acid	[3]
$C_{18}H_{34}O_2$ (18:1) 	Oleic acid	Fatty acid	[3]
$C_{18}H_{32}O_2$ (18:2) 	Linoleic acid	Fatty acid	[3]
$C_{18}H_{30}O_2$ (18:3) 	Linolenic acid	Fatty acid	[3]
	Persenone A	Acetogenin	[32]
	Persenone B	Acetogenin	[32]
	Cyanidin 3-***O***-glucoside	Anthocyanin	[22]

In a later publication [25], the same group reported that metabolic profiling using the GC-APCI-TOF MS platform combined with profile analysis by PCA and PLS-DA provided a very powerful tool through which to achieve a better understanding of the effect that the ripening process has on the contents of different avocado varieties. Using this technique, 15 metabolites, including aspartic, malic, citric, stearic and pantothenic acids, mannoheptulose, mannitol, pentadecylfuran, β-sitosterol and persenone, were found to be most influential according to the PLS-DA model. In a further extension to this

work, the value of GC-EI-quadrupole-MS as a non-targeted profiling approach for the evaluation of biological changes in the metabolic composition of avocado fruits was assessed [26]. The method allowed the identification of some 60 metabolites in a single run, among which mannoheptulose and *p*-coumaric, eicosenoic, aspartic and abscisic acids were selected as the most influential. Further filtering using a two class PLS-DA model identified mannoheptulose and abscisic, linoleic and aspartic acids as compounds with the highest influence.

An alternative methodology involving capillary zone electrophoresis (CZE) coupled with targeted multiple reaction monitoring (MRM) MS and non-targeted full-scan MS has been used to study biochemical changes during the development and ripening of the avocado fruit [27]. Ten metabolites representing six different chemical classes were quantified and two phenolic acid-related sugars that changed their levels during ripening were identified.

The advantages and limitations of the various metabolomic platforms used in evaluating the complex matrix of components present in the fruit have been reviewed [28]. It was concluded that ultra HPLC-UV-ESI-TOF-MS may be the method of choice for metabolomic studies with the avocado fruit by virtue of its capacity to determine different classes of compounds with high sensitivity, reproducibility and speed. Using this technique it was possible to quantify a number of metabolites whose concentrations varied depending on the avocado variety and stage of ripeness, namely quinic acid (with 18.95 mg/kg in one unripe sample), succinic and pantothenic acids (with 176.88 and 6.14 mg/kg, respectively, in ripe samples) and epicatechin (with 26.72 mg/kg in a sample at the first stage of ripeness), the last mentioned being of particular note since this flavonol is difficult to quantify. Interestingly, although CZE coupled with UV-MS was the least informative platform in terms of the number of metabolites identified, it was considered to be a powerful and complementary technique for the rapid analysis of polar compounds.

A limited number of reports relate to the post-harvest physiological and biochemical intra-varietal differences of avocado fruits from different suppliers. It has been demonstrated, for example, that the main bioactive compounds present in the mesocarp of 'Hass' avocado depend strongly on the origin of the fruit and its consistency during ripening, while the principal fatty acids of the mesocarp oil are determined by pre-harvest factors rather than post-harvest conditions [29]. The results obtained in this study also supported the hypothesized role of C7 sugars, including D-mannoheptulose, in the ripening process of the avocado fruit regardless of fruit origin and/or harvest time.

It would appear that the popular 'Hass' cultivar harbors a particularly complex physiology such that its post-harvest ripening is both heterogeneous and unpredictable. However, only limited information is currently available on the evolution of relevant metabolites present in the fruit from the time when it is picked until the moment it is ready for consumption. Pedreschi *et al*. [30] tried to solve this problem by analyzing mesocarp tissue using a fruit biopsy method with targeted and non-targeted metabolomic approaches. Non-targeted profiling of polar and semi-polar compounds revealed several metabolites, mainly amino acids and fatty acids, that might be indicative of differences in the age of the fruit and/or other pre-harvest factors related to ripening heterogeneity. Hurtado-Fernández *et al*. [31] described an alternative strategy involving GC with simultaneous flame ionization detection (FID) and APCI-MS detection to quantify 27 metabolites of different chemical classes, including organic acids, phenolic acids, flavonoids, phytohormones and vitamins, in 13 varieties of avocado at two different ripening stages. The results obtained using the two detectors were compared and PCA was applied in order to assess the influence that the quantified compounds had on the differentiation between varieties and ripening stages. The results showed that the levels of organic acids and flavonoids decreased during ripening while the concentrations of hydroxycinnamic acid derivatives and vitamin B_5 exhibited an opposite trend. The authors concluded that GC-FID remains a useful tool by which to determine some of the most common metabolic markers that discriminate between ripe and unripe samples and/or cultivars of avocado.

Although fatty acids, phenolic compounds, organic acids and vitamins have generally been considered as the main indicators of fruit ripeness

Fig. 1. Characteristics of three landraces of avocado showing: a) Mexican b) West Indian and c) Guatemalan.

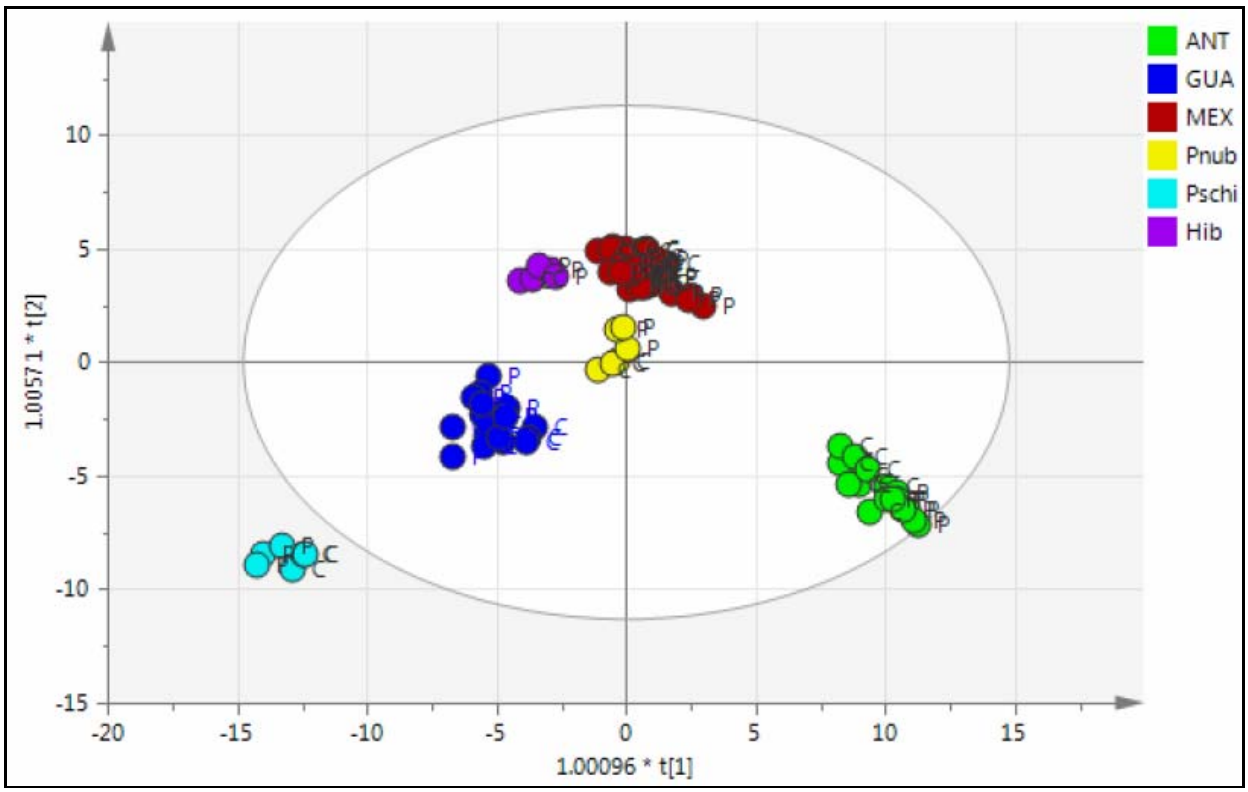


Fig. 2. OPLSD of avocado races and related species. P = mesocarp, c = skin, WES = West Indian race, GUA = Guatemalan race, MEX = Mexican race, PNUB = *Persea nubigena*, PSchi = *Persea schiedeana*; Hyb = hybrid.

in avocado, knowledge of the acetogenin content could also contribute to our understanding of the complex matrix of the fruit. The acetogenins present in avocado are derivatives of fatty acids with an odd-carbon aliphatic chain and an acetoxy group that contributes two additional carbons. Rodríguez-Sánchez *et al.* [32] related the acetogenin content of avocado seeds to their antimicrobial properties and found that three compounds, namely, persenones A, B and C, inhibited endospore germination and vegetative growth in *Clostridium sporogenes*. The authors concluded that these acetogenins could represent natural alternatives to the agents currently employed to protect food and pharmaceuticals against Gram-positive spore-forming bacteria.

Rodríguez-López *et al.* [33] characterized and quantified the acetogenins in 22 varieties of avocado using a targeted metabolomic approach with the aim of determining the natural variation

of this class of compound. Eight acetogenins were identified, all of which were present in almost all of the cultivars screened. However, while the peel and pulp of the fruits exhibited similar profiles, those of the seed material were differentiated by the accumulation of different acetogenins. The authors concluded that acetogenin metabolism is conserved in avocado and that the acetogenin profile can contribute to the assignment of landraces to plants of unknown origin, thereby assisting breeders in parental selection.

In addition to the metabolites mentioned earlier, avocado also contains rare sugars such as D-mannoheptulose and perseitol, which have been described as storage materials and as indicators of the progress of ripening [34-36].

In this context, Ibarra-Estrada [37] explored the use of ^{1}H-NMR as an analytical tool by which to determine the landrace or natural species from which a fruit is derived. The three horticultural races of avocado common in Mexico, namely Mexican, West Indian and Guatemalan (Fig. 1), were classified on the basis of data obtained using the ^{1}H-NMR platform, and application of an orthogonal PLS-DA model helped to highlight differences and similarities between the races. The West Indian race was characterized by the accumulation of sugars and amino acids and was grouped apart from the Mexican and Guatemalan races, which presented variable concentrations of these primary metabolites and of the co-occurring aromatic compounds. The relationship between these races, *P. nubigena* and *P. schiedeana*, and the Hass hybrid are represented in Fig. 2.

CONCLUSION

In order to understand the physiological effects mediated by the consumption of avocado fruits, it is necessary to have a detailed knowledge of the chemical composition of the tissue and to relate this with biological activity. In this sense, metabolomics is an ideal analytical tool by which such information can be gathered and processed.

Analysis of the main features of metabolomic studies carried out on avocado fruits and published since 2000 revealed that most have employed metabolic profiling with the aim of identifying biomarkers related to fruit growth, ripening, classification or storage, to understand the metabolic shifts that occur during fruit development and to establish the genetic architecture underlying the accumulation of metabolites. The lipophilic fraction of avocado fruits and the metabolic profiles related to the stage of ripeness have been most extensively studied. A body of evidence suggests that mannoheptulose, perseitol or several of the acetogenins present in avocado could be used as indicators of fruit maturity. It is expected that metabolomics, along with other 'omic' technologies, will be developed in order to solve the puzzle of the regulatory and signaling pathways responsible for triggering and coordinating fruit maturation. Detailed knowledge of these pathways would lead to efficient and affordable methods to extend the shelf-like of fresh fruits such as avocado.

Metabolomic approaches can also be applied to processed foodstuffs, including those prepared using fruit materials, for the purpose of checking quality, identifying constituents, establishing authenticity and determining geographical origin (traceability). Moreover, metabolic profiling is a powerful tool for testing the nutritional equivalence of novel crops. It is likely that future metabolomic studies involving avocado will focus more on these aspects as consumers exhibit greater critical concern regarding industrialized food and drink products.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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